INTERNATIONAL SEARCH REPORT

Int[illegible]ional Application No
PCT/CA 00/00445

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N7/01 C12N15/86 C12N15/85 C12N15/63 C12N5/10
A61K48/00 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, PAJ, EPO-Internal, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Nalbantoglu J. et al.:"VSV-G pseudotyped retrovector mediates high efficiency in vivo gene transfer in glioma-targeted suicide gene delivery" NEUROLOGY, vol. 52,12 April,1999 (1999-04-12), page A425 XP000964616 the whole document ---	1-9, 13-15
X	WO 99 04026 A (CHIRON CORP) 28 January 1999 (1999-01-28) page 2, line 5 - line 21; claims 1,3,5,6 page 13, line 8 - line 21 page 17, line 1 - line 4 page 46, line 12 - line 18 --- -/--	1-6,10, 12,13,16

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 November 2000	20/11/2000
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Schönwasser, D

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/CA 00/00445

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	GALIPEAU J. ET AL.: "Vesicular stomatitis virus G pseudotyped retrovirus mediates effective in vivo suicide gene delivery in experimental brain cancer" CANCER RESEARCH, vol. 59, 15 May 1999 (1999-05-15), pages 2384-2394, XP000926033 the whole document	1-17
A	ORY D S ET AL: "A STABLE HUMAN-DERIVED PACKAGING CELL LINE FOR PRODUCTION OF HIGH TITER RETROVIRUS/VESICULAR STOMATITIS VIRUS G PSEUDOTYPES" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 93, no. 21, 15 October 1996 (1996-10-15), pages 11400-11406, XP002030515 ISSN: 0027-8424 the whole document	1-17

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. ional Application No
PCT/CA 00/00445

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9904026 A	28-01-1999	AU 8576298 A	10-02-1999
		EP 1003894 A	31-05-2000

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
14226-2PCT		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/CA 00/ 00445	20/04/2000	23/04/1999
Applicant		
CENTRE FOR TRANSLATIONAL RESEARCH IN CANCER et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[ ] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[X] furnished subsequently to this Authority in written form.

[X] furnished subsequently to this Authority in computer readble form.

[X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

[ ] None of the figures.

[ ] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

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PCT/CA 00/00445

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 November 2000	20/11/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Schönwasser, D

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International Application No
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		EP 1003894 A	31-05-2000

XP-000964616

P05.078

VSV-G Pseudotyped Retrovector Mediates High Efficiency *In Vivo* Gene Transfer In Glioma-Targeted Suicide Gene Delivery

Josephine Nalbantoglu, Hewei Li, Andre Paquin, Franka Sicilia, George Karpati, Jacques Galipeau, Montreal, QC, Canada

p. 71

p.d 12-4-1999

p. A425 (1)

OBJECTIVE: To determine whether a retrovector expressing the herpes simplex virus (HSV) thymidine kinase (TK) gene can be utilized for efficient and tumor-specific TK gene delivery *in vivo* upon pseudotyping with the Vesicular Stomatitis Virus G protein (VSV-G).

BACKGROUND: Cancer cells engineered to express the HSV TK gene are sensitized to a toxic effect of the prodrug gancyclovir (GCV). This forms the basis for the strategy of "suicide" gene therapy of cancer. Direct *in vivo* tumor targeting with replication-defective TK viral vectors is limited by either inefficient gene transfer (i.e. retroviral vectors) or indiscriminate transfer of the transgene to adjacent non-malignant tissue (i.e. adenoviral vectors). VSV-G pseudotyped vectors differ from standard murine retroviral pseudotypes by their broad tropism and by their physical stability. VSV-G retrovectors can be frozen-thawed and concentrated by ultracentrifugation without loss of activity. These properties may make the VSV-G pseudotyped TK-expressing retrovector an efficient delivery system for targeting tumors *in vivo*.

DESIGN/METHODS: A novel bicistronic retroviral vector (vTKiGFP) was developed which expresses TK and EGFP (Enhanced Green Fluorescent Protein). The EGFP serves as a reporter of provirus transfer and expression in target cells. Supernatant collected from stably transfected polyclonal vTKiGFP producer cell line was concentrated a 1000-fold by ultracentrifugation, raising the titer from 2.9×10^7 cfu/ml to 2.3×10^{10} cfu/ml. Concentrated retrovector stock was injected stereotactically into pre-established tumors formed by implantation of C6/lacZ glioma cells into rat brain. Subsequently, rats were treated daily with GCV (i.p.) for a period of 10 days.

RESULTS: Control rats (tumor+, retrovector+, but no GCV; n = 5) had a mean survival of 38 days (range 20–52 days). Sections of post-mortem brain tissue revealed large tumors with evidence of high efficiency retrovector transfer and expression (as assessed by GFP fluorescence). Fluorescence was restricted to malignant tissue. In the experimental group (tumor+, retrovector+ and GCV; n = 12), two animals died early on likely from GCV toxicity; of the others, 8 out of 10 remain alive and well >120 days post tumor implantation.

CONCLUSION: VSV-G pseudotyped retroviral particles can be concentrated to titers which allow direct intra-tumoral delivery *in vivo*. The high multiplicity of infection which is achieved leads to biologically significant TK expression. The therapeutic efficiency of the direct injection of this retrovector is demonstrated by the significantly increased survival of animals with intracerebral experimental gliomas.

Supported by: Medical Research Council of Canada and National Cancer Institute of Canada

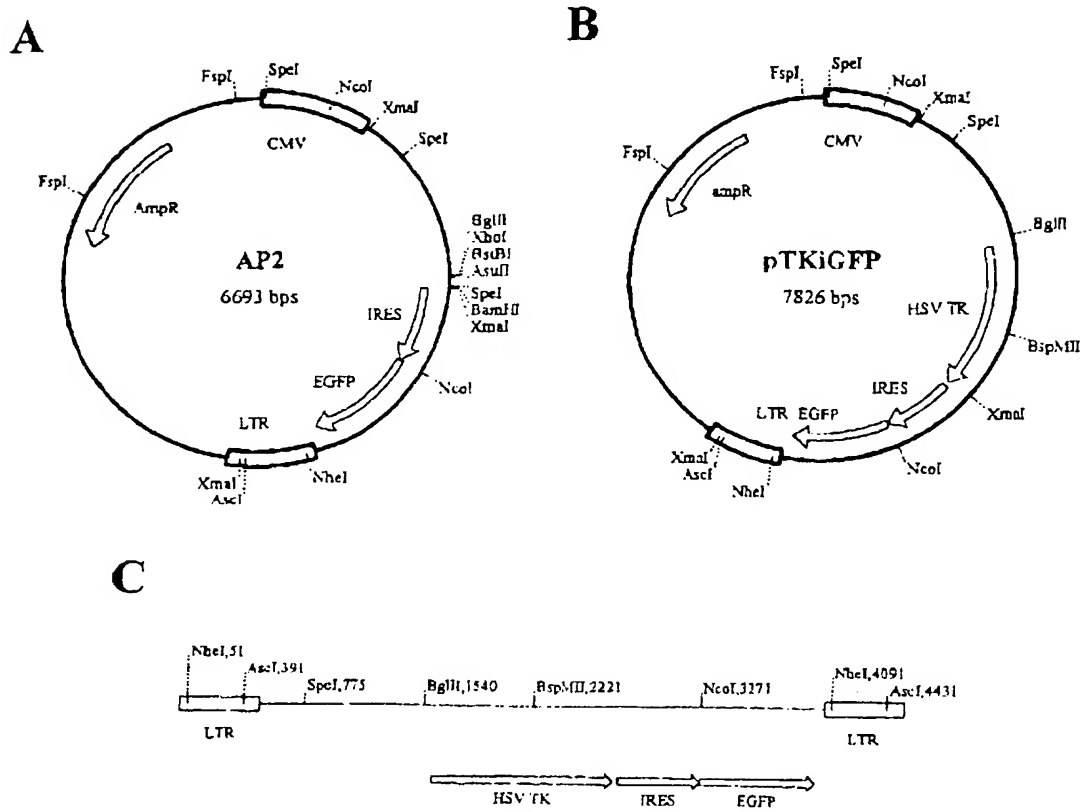

Fig. 1. Schematic representations of plasmids and retrovectors. *A*. AP2 plasmid retrovector serves as a template for the coexpression of the EGFP reporter and of a linked cDNA in eukaryotic cells. The cDNA of interest is inserted in the multiple cloning site upstream of the IRES. *B*. pTKiGFP is a derivative of AP2, which contains the HSV *TK* gene. Transfection of this plasmid into retroviral packaging cells will lead to the production of replication-defective retroparticles. *C*. target cells transduced with vTKiGFP will integrate the retrovector in their genomic DNA. The DNA structure (flanked by LTRs) and coding sequences are depicted.

$$\% \text{ survival} = \frac{A_{570} \text{ test} - A_{570} \text{ empty well}}{A_{570} \text{ untreated cells} - A_{570} \text{ empty well}} \times 100$$

All data points were measured in triplicate in at least three separate experiments.

Titration of Retrovector. Target glioma cells were plated at 2×10^5 cells/well in a six-well tissue culture dish. The next day, cells from a test well were trypsinized and enumerated to determine baseline cell count at the moment of virus exposure. Virus was serially diluted (range, 100 to 0.001 μl) in a final volume of 1 ml of RPMI/10% FBS supplemented with 6 μg/ml polybrene (Sigma) and applied to adherent cells. Flow cytometric analysis was performed 3 days later to determine the percentage of GFP+ cells. Viral titer (cfu/ml) was extrapolated from the test point in which nonsaturating transduction conditions prevailed (*i.e.*, transduction efficiency <80%). Titer (cfu/ml) was calculated as:

$$\text{Titer} = \frac{\% \text{ GFP}+ \text{ cells} \times \text{cell number at initial viral exposure}}{\text{Viral volume in ml applied}}$$

Animal Model of Brain Cancer, *in Vivo* Retrovector Delivery, and GCV Treatment. C6/lacZ glioma cells will reproducibly generate lethal intracerebral tumors when injected in Sprague Dawley rats. The constitutive β-galactosidase expression facilitates delineation (by X-gal staining) of tumor cells and extent of the tumor infiltrate in postmortem brain sections. Adult Sprague Dawley rats were anesthesized with i.p. injection of ketamine (50 mg/kg) and xylazine (2 mg/kg). C6/lacZ rat glioma cells (2×10^4 cells in 5 μl of HBSS) were injected intracranially into the frontal lobe using a Hamilton syringe in a stereotactic apparatus (Kopf) over a period of 15 min. The coordinates used were 3.5 mm lateral to the bregma, 1.0 mm posterior to the coronal plane, and 4.5 mm in depth of the dural surface. Six days after glioma cell implantation, rats were anesthesized, and vTKiGFP (concentrated stock of 2.3×10^{10} cfu/ml) was injected of into six different sites (1 mm apart) in the preestablished tumor guided by the previous stereotactic coordinates. A total volume of 9 μl was injected in each tumor (6×1.5-μl increment), and the needle was left in place for at least 5 min/increment (for a total of 30 min/tumor). Two days after retrovector delivery, rats are treated with 50 mg/kg GCV i.p. twice daily for 5 days, followed by 50 mg/kg once daily for another 5 days. After euthanasia, brains were removed and quickly frozen in isopentane chilled with liquid nitrogen. Coronal sections (10 μm) were prepared. GFP activity was observed by epifluorescence microscopy and recorded photographically. Subsequently, sections were stained histochemically for β-galactosidase activity as described previously (41) before counter staining with H&E.

RESULTS

Retrovector Design and Synthesis. The AP2 expression vector (Fig. 1*A*) allows the incorporation of a cDNA sequence in a Multiple cloning site upstream of an IRES and the EGFP cDNA. The transcription initiation from a cytomegalovirus promoter will lead to the production of a bicistronic mRNA incorporating both the inserted cDNA and the EGFP coding sequence. Translation of both coding sequences will be achieved from a single mRNA molecule, thereby ensuring codominant expression of both protein products. Live cells expressing EGFP, which is detectable by fluorescence microscopy or flow cytometry, will coexpress the linked gene product. Gene-modified cells can be implanted or transplanted in animal models, and their localization and function can be traced based on the expression of the

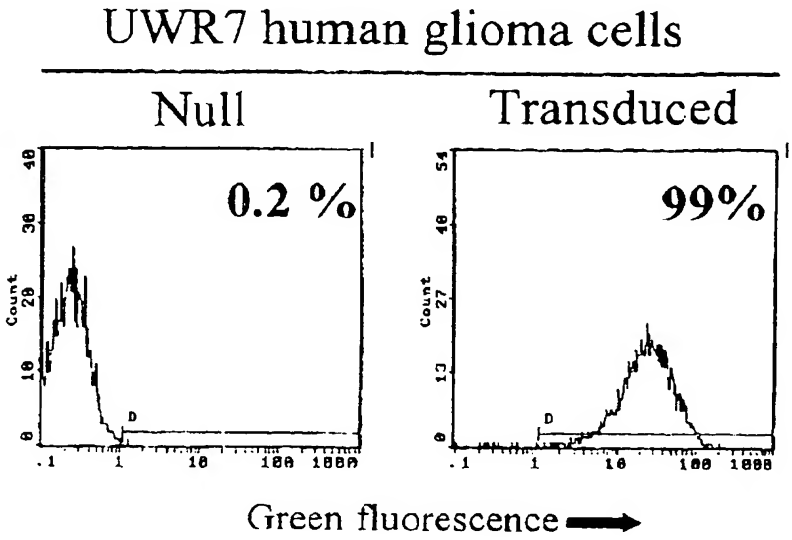

Fig. 2. Flow cytometric analysis of vTKiGFP-transduced glioma cells. UWR7 human glioma cells were transduced with vTKiGFP and subsequently analyzed by flow cytometry for green fluorescence, as described in "Materials and Methods." GFP serves as a reporter of retrovector expression in transduced cells.

EGFP protein. The AP2 expression vector incorporates a replication-defective retroviral packaging sequence and a retroviral 3′ LTR. Transfection of an appropriate retroviral packaging cell line can lead to production of recombinant retroviral particles. Retroparticles can be generated either by transient transfection of packaging cell lines, or alternatively, stable producer cell lines can be generated by cotransfection with a drug resistance plasmid. We have generated retroparticles by both methods with good success using the 293GPG retroviral packaging cell line.

Retrovector Transfer and Expression in Human Glioma Cell Lines. The 293GPG packaging cell line was transiently transfected with pTKiGFP (Fig. 1*B*), and supernatant containing VSVG-typed retroparticles (vTKiGFP) was subsequently collected, filtered, and frozen for storage. Human glioma cell lines (SKI-1, SKMG-4, SKMG-1, T98G, UW28, and UWR7) were transduced with three consecutive daily applications of thawed vTKiGFP supernatant. The MOI was 4 at each viral application. Six days after transduction, polyclonal cell lines were subjected to flow cytometric analysis to determine the proportion of cells that expressed the GFP reporter protein. All polyclonal cell lines were 100% GFP-positive by fluorescence-activated cell sorting analysis, and transduced UWR7 cells served as a representative example (Fig. 2). We have also found that GFP expression could be easily detected in live cultured cells by direct visualization with a tissue culture microscope fitted with an epifluorescence light source (data not shown). Southern blot analysis confirmed that unrearranged vTKiGFP vector integrated in chromosomal DNA of transduced target cells (Fig. 3). vTKiGFP transduced cells have been passaged in excess of 30 times without loss of GFP expression.

vTKiGFP Expression and GCV Sensitization. HSV TK expression will lead to the conversion of the prodrug GCV to its cytotoxic metabolite GCV monophosphate. Cells that do not express this enzyme are refractory to GCV toxicity. We compared the GCV sensitivity of vTKiGFP transduced cells with unmodified parental cells as well as cells modified with a control, GFP-containing retrovector (vMSCV-DIG). Cells were plated in 96-well dishes and exposed to GCV for a period of 6 days. Live cell content was assessed colorimetrically by MTT assay, and cell survival was expressed as a percentage relative to untreated cells. We have found that all vTKiGFP-expressing cell lines were sensitized to GCV. Comparing the GCV concentration that inhibits cell growth by 50% (IC_{50}), we found that vTKiGFP-transduced cells (all six cell lines aggregated) were up to 10,000-fold more sensitive to GCV than controls (IC_{50} tests, 0.004 μg/ml *versus* IC_{50}controls; 40

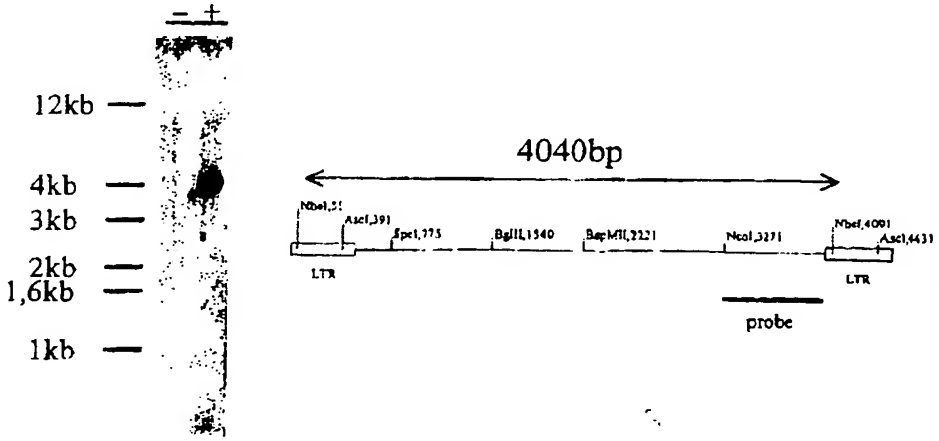

Fig 3. Southern Blot analysis on vTKiGFP-transduced glioma cells. After transduction with vTKiGFP, the retrovector will integrate into genomic DNA. Digest of genomic DNA with *NheI*, which cuts once in each flanking LTR, and subsequent probing of Southern blot with a vector complementary sequence will allow detection of integrated proviral sequences with a predicted size of 4 kb (*right*). *Left*, Southern blot analysis of transduced (+) and untransduced (−) UWR7 cells with a GFP cDNA-specific probe, as described in "Materials and Methods." Molecular weights are indicated.

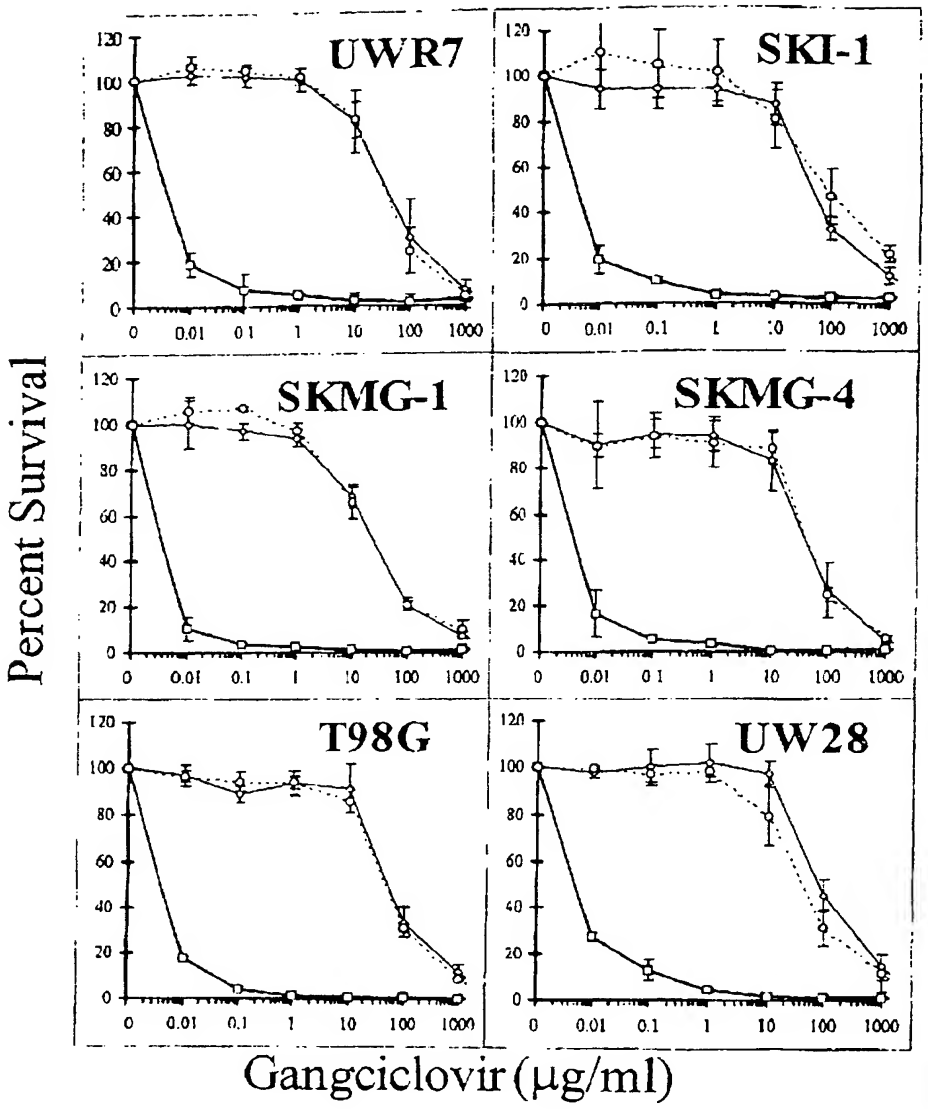

Fig. 4. Growth suppression of human glioma cells with GCV. The indicated human glioma cell lines were transduced with vTKiGFP (□) or the control retrovector vDHFRiGFP (○). These and untransduced controls (●) were subsequently exposed to GCV for 6 days, and cell survival was measured by the MTT assay as described in "Materials and Methods." Percent survival is plotted against GCV concentration (log scale). *Data points*, mean survival measured in three separate experiments, *bars*, SD. SD smaller than data point icon are not displayed.

μg/ml: $P < 0.001$ by Student's t test; Fig. 4). Growth rates for transduced and parental cell lines in the absence of GCV were identical (data not shown).

Concentration of vTKiGFP Retroparticles. The most direct means of transducing a tumor *in vivo* is to inject the therapeutic retrovector intratumorally. If the aim is to transduce as many tumor cells as possible, it would be desirable to inject a concentrated vector stock to achieve a high local MOI. We determined whether viable vTKiGFP retroparticles could be concentrated by ultracentrifugation as described previously (31). As a first step, we transfected 293GPG cells with pTKiGFP and a Zeocin resistance plasmid (pJ6bleo). A stably transfected, Zeocin-resistant polyclonal producer cell population (293AP3) was generated. Flow cytometric analysis for GFP fluorescence revealed that 42% of this mixed population stably expressed the pTKiGFP vector DNA (Fig. 5). Tetracycline withdrawal from the culture media will lead to the production of VSVG-typed vTKiGFP retroparticles. We collected retroparticle-containing media daily from the 293AP3 producer cells from days 3 to 8 after tetracycline withdrawal. Supernatant was cleared of cellular debris with a 0.45 μm filter and frozen. We have noted that twice daily media collection, as opposed to once daily, doubled the yield of retroparticles from producer cells after tetracycline withdrawal (data not shown). Media were thawed, pooled, and subjected to ultracentrifugation as described in "Materials and Methods." Supernatant was concentrated 84-fold (20–0.24 ml) by ultracentrifugation. The concentration step raised the titer from 2.9×10^7 cfu/ml to 220×10^7 cfu/ml, as measured on UWR7 human glioma cells (Fig. 6). Concentrates (84×) were pooled and subjected to a second ultracentrifugation to achieve a final 1000× (100-ml initial volume to 0.1 ml final volume) concentration. Titer of 1000× retrovector was 2.3×10^{10}, as determined on rat C6 glioma cells (Fig. 6). Concentrated retrovector aliquots were stored at −80°C until further use. We have observed that unmanipulated (unconcentrated) supernatant from tetracycline-deprived 293GPG producer cells can be toxic to target cells if applied repeatedly. However, no toxicity was observed on target cells if concentrated supernatant was used for transduction purposes, even at the highest tested MOI (>100).

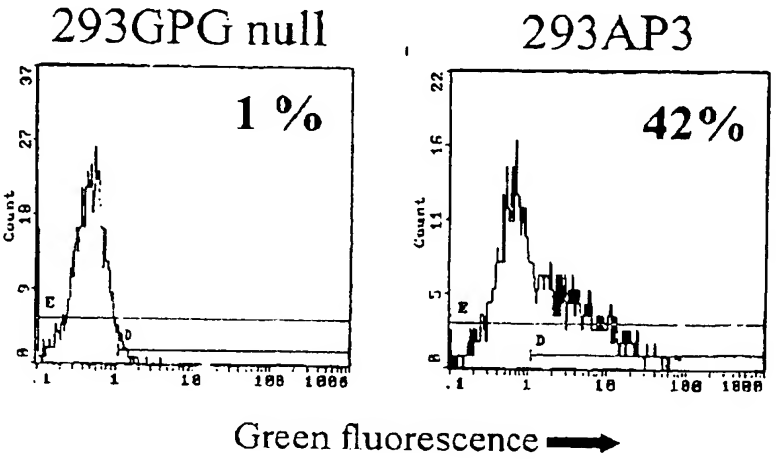

Fig. 5. Flow cytometric analysis of 293AP3 producer cells. 293GPG packaging cells were stably transfected with pTKiGFP and a Zeocin resistance plasmid. A mixed population of Zeocin-resistant 293AP3 cells was generated and characterized for GFP expression by flow cytometry as described in "Materials and Methods." The percentage of GFP + cells is indicated. These cells were subsequently used to generate vTKiGFP stock for concentration and *in vivo* delivery.

Retrovector Expression after Intratumoral Injection of Concentrated vTKiGFP Retroparticles. Implantation of C6/lacZ glioma cells will reliably lead to the establishment of intracerebral tumors in immunocompetent Sprague Dawley rats. This cell line will generate large local tumors that are uniformly lethal within 60 days after the initial stereotactic injection of 2×10^4 cells. Furthermore, C6/lacZ cells constitutively express β-galactosidase, which permits the assessment of tumor extent and local invasion in X-gal-stained postmortem brain sections. Eighteen rats received 2×10^4 C6/lacZ cells via stereotactic injection in the right brain hemisphere. Six days later, 9 μl of 1000× vTKiGFP retrovector (2×10^{10} cfu/ml) were injected at the tumor site using the same stereotactic coordinates. Of these 18 rats, 6 were randomly chosen and treated with saline. Saline-treated control rats had an average survival of 38 days (range, 20–52 days). Postmortem examination of brain revealed macroscopic intracerebral tumors, except for one rat, which died with leptomeningeal tumor spread 8 days after tumor injection (which was excluded from further analysis). Examination of fresh frozen brain sections by epifluorescence microscopy shows that in all animals, a predominant proportion of glioma cells fluoresce green (Fig. 7*A*), including distant micrometastasis. Normal surrounding brain tissue is bereft of green fluorescence. No green fluorescence was observed in untransduced brain tumors (Fig. 7*C*).

GCV Treatment of Rats with vTKiGFP-targeted Gliomas. Of 18 rats having received intratumoral vTKiGFP retrovector, 12 were subsequently treated with GCV. Two days after retrovector injection, rats received 50 mg/kg GCV i.p. twice daily for 5 days, followed by 50 mg/kg once daily for another 5 days. Significant GCV toxicity, including transient limb paresis and otorhagia, was noted in some rats in the week after GCV treatment. Of 12 GCV-treated rats, two died within 10 days after drug treatment, presumably from direct GCV toxicity (both animals had brain tumors <1 mm in diameter on postmortem). The other 10 rats fully recovered from GCV toxicity. Two rats developed tumor relapses at the initial injection site and died of progressive disease at day 82. Examination of brain tissue sections on these late relapses, revealed focal GFP expression in the tumors (Fig. 7*E*). Significantly enhanced survival was obtained; 8 of 12 GCV-treated test rats (66%) remain long-term survivors (>120 days). A supplementary control cohort of six rats implanted with C6/lacZ, but without subsequent retrovector administration, was treated with the same GCV regimen. These controls had an average life span of 47 days (range, 31–63 days; Fig. 8). With our experimental C6 glioma model, we have not observed a significant difference in average survival between the two control groups [saline controls *versus* GCV-treated null tumors, $P = 0.37$ (Student t test)], suggesting that GCV treatment, on its own, does not have a measurable impact on survival, as has been suggested by others using 9 L glioma implants (42). These differences may be due different biological properties of these two experimental glioma models.

DISCUSSION

Engineering tumor cells to express the HSV *TK* cDNA will lead to their destruction if they are subsequently exposed to nontoxic nucleobase analogues such as GCV. This "suicide" effect is accompanied by "bystander" toxicity on adjacent tumor cells not expressing TK, so that a minority of engineered tumor cells, perhaps no more than 10–25%, will lead to 100% tumor eradication (5, 15, 43). Clinical application of this therapeutic strategy requires relatively high efficiency *TK* gene transfer to preestablished tumors. Furthermore, "collateral" gene transfer to normal adjacent normal tissue would have to be curtailed to prevent GCV toxicity to normal brain tissue.

The affinity of recombinant retroparticles for target tissue is defined by the *env* protein. Murine amphotropic retroviruses, from which are derived many of the therapeutic retrovectors in glioma-targeted gene delivery, will only bind target cells that express a specific inorganic phosphate transporter (29). If a target tumor does not express the retrovirus receptor, gene transfer–and therapeutic benefit–is unlikely to occur. Retroparticles that are pseudotyped with the VSVG protein will adopt the wide host range of the VSV. The putative VSVG receptor on target cells, which is believed to be membrane phospholipid (44), is ubiquitously found in all eukaryotic cells. This has led to the use of VSVG-pseudotyped retrovectors as gene delivery vehicles in a wide assortment of mammalian, nonmammalian, and invertebrate cells (31, 45–49). A major advance in pseudotyping retrovectors with VSVG was achieved when a practical "transient" VSVG retroviral packaging cell line was designed (31). The subsequent publication of reliable "stable" high-titer VSVG packaging cell lines (50, 51), including 293GPG (34), has allowed the development and characterization of pseudotyped retrovectors for a wide variety of gene transfer applications (52), including tumor cell-targeted gene delivery (53).

We have examined the utility of a VSVG-pseudotyped suicide retrovector for glioma-targeted gene delivery. To facilitate analysis of vector transfer efficiency and expression in target cells, we engineered a retroviral expression vector that incorporates HSV TK and the EGFP reporter cDNA within a bicistronic transcript (pTKiGFP). We have found that codominant expression of the HSV TK cDNA and of the EGFP reporter facilitates a wide assortment of procedures asso-

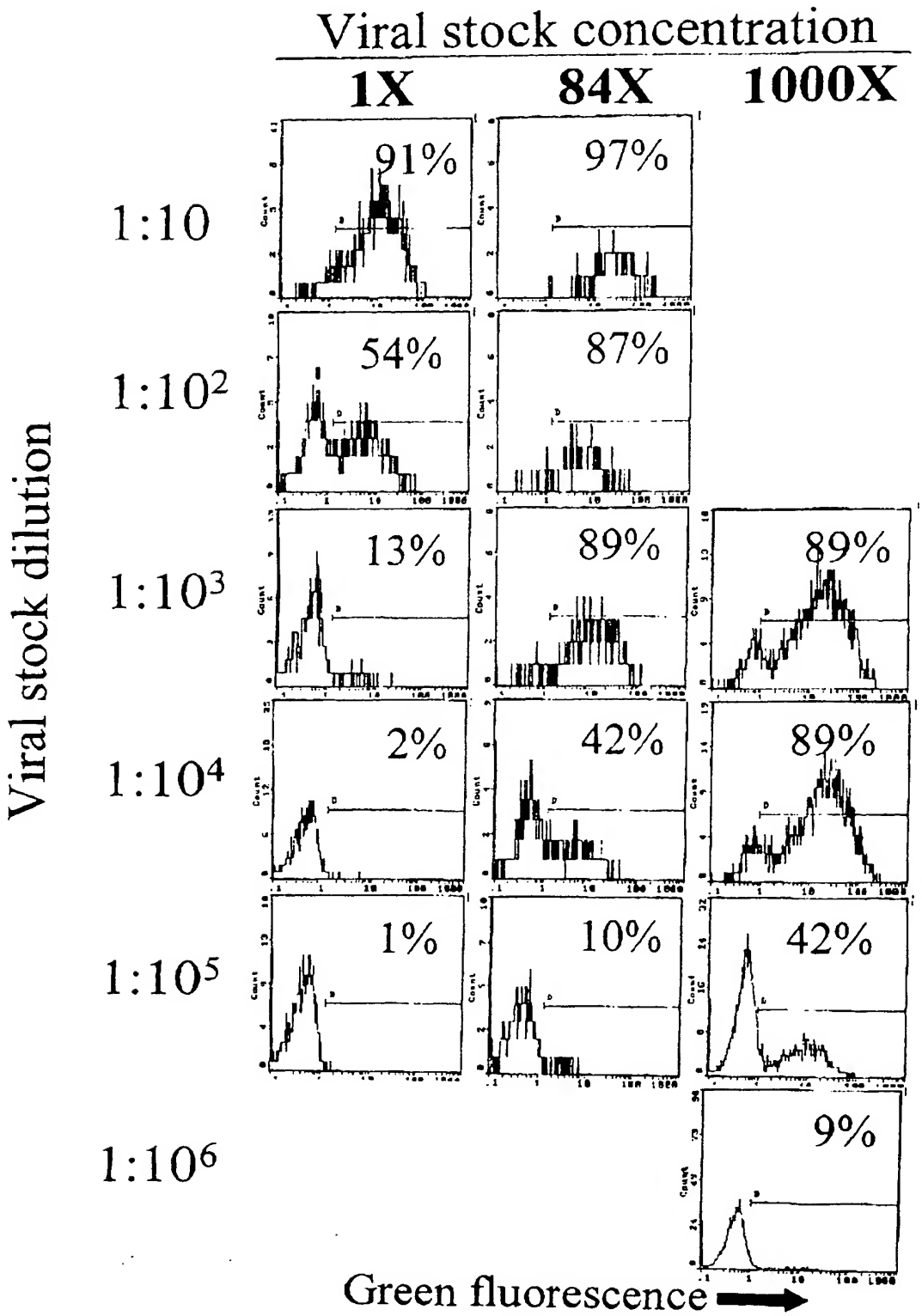

Fig 6. Transduction of glioma cells with concentrated vTKiGFP retrovector stocks. vTKiGFP retroparticles were collected and concentrated to 84- and 1000-fold (volume/volume) as described in "Materials and Methods." Virus stocks (1× and 84×) were diluted (*left*) in a final volume of 1 ml and applied to 2.3×10^5 UWR7 cells in a 24 well dish. Three days after a single application of vector, cells were analyzed for GFP expression by flow cytometry. Percent GFP+ is indicated in histogram figures. Dilutions of 1000× stock were applied to 5.4×10^5 C6 glioma cells and analyzed 3 days later for GFP expression. Titers extrapolated from these experiments were: 1×, 2.9×10^7 cfu/ml; 84×, 2.2×10^9 cfu/ml; 1000×, 2.3×10^{10} cfu/ml.

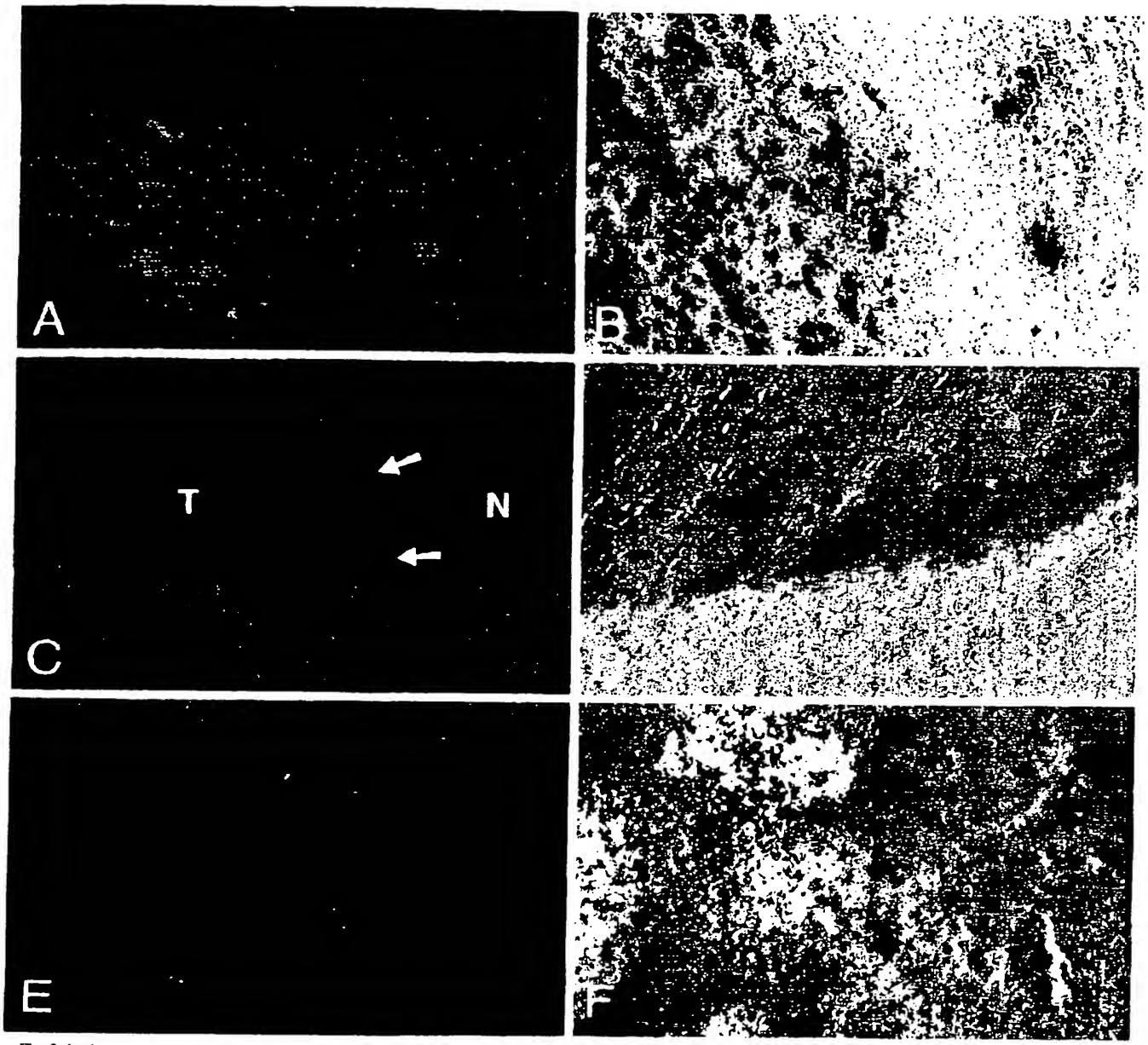

Fig. 7. *In vivo* transduction of C6/lacZ tumors with vTKiGFP. Brain tumors were harvested postmortem as described in "Materials and Methods." *A* and *B*, tumor from a control rat that received vTKiGFP without subsequent treatment with GCV (rat was sacrificed on day 30 after tumor implantation due to morbid state). *C* and *D*, tumor from a control rat that did not receive vTKiGFP but was treated with GCV (rat was sacrificed on day 43). *E* and *F*, tumor from a test rat that received vTKiGFP and subsequent treatment with GCV, which suffered symptomatic recurrent tumor (rat was sacrificed on day 82). GFP expression (*A*, *C*, and *E*) was compared with subsequent histochemical staining of C6/lacZ tumor cells with the substrate X-gal (*B*, *D*, and *F*). ×100. *T*, tumor; *N*, normal brain.

ciated with synthesis and characterization of viral vectors. Among these are the ability to measure end point titer from stable retroviral producer cells (Fig. 6) as well as potential use for selecting GFP+ producer cells with a cell sorter device. We have also found that the EGFP reporter can serve as a sensitive marker of retrovector expression in targeted tissue *in vitro* (Fig. 2) as well as *in vivo* (Fig. 7).

We generated a stable retroviral vTKiGFP producer cell line (293AP3) derived from the 293GPG packaging cell line (Fig. 5). Upon tetracycline withdrawal, this retroviral producer cell line will express the VSVG envelope protein and generate pseudotyped retroviral particles. We found that VSVG-pseudotyped retroparticles incorporating vTKiGFP will lead to high efficiency retrovector transfer to human glioma cell lines *in vitro*. In contrast with standard transfection techniques, or with the use of more "standard" retroviral pseudotypes, we have not required dominant selection of subpopulations of cells to achieve 100% transgene-positive cell populations. Retroparticle-conditioned media collected from 293GPG cells transiently transfected with pTKiGFP was used to generate vTKiGFP-transduced glioma cell lines. We noted that transducing glioma cells with concentrated retrovector with a single application at a MOI of ~5 led to >90% gene transfer in targeted cells (Fig. 6). Gene expression was durable as assessed by persistent GFP expression

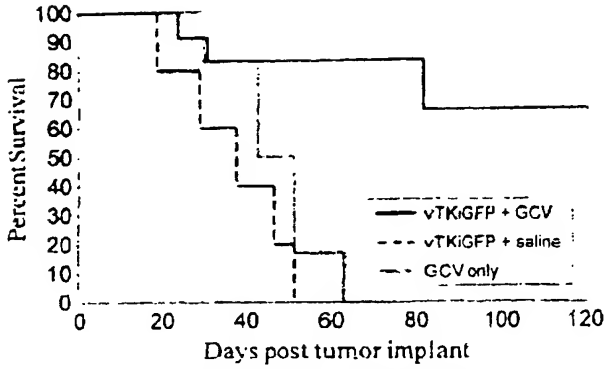

Fig. 5. Kaplan-Meier survival curve of rats with experimental glioma. Sprague Dawley rats received 2×10^4 C6/lacZ glioma cells by stereotactic injection in the right brain hemisphere as described in "Materials and Methods." Six days later, 18 animals were administered 9 μl of 1000× vTKiGFP stock in the same stereotactic coordinates as the previous C6/lacZ implant. Forty-eight h later, test animals ($n = 12$) received 50 mg/kg GCV twice daily for 5 days, followed by 50 mg/kg once daily for 5 more days. The other animals ($n = 6$) were administered saline only. In a separate experiment, a supplementary cohort ($n = 5$) received a C6/lacZ glioma implant, followed 9 days later by GCV treatment (no retrovector administered). The survival seen in the test group (vTKiGFP + GCV) is significantly greater than that in either control groups ($P < 0.001$ by Log rank). There is no significant difference in survival between the two control groups.

(>30 passages) and by functional HSV TK expression, rendering VSVG-associated pseudotransduction (46, 54) unlikely. Having generated vTKiGFP-transduced cell lines, we confirmed that the proviral genome integrated unrearranged by Southern analysis, demonstrating the stability of the viral vector as designed (Fig. 3). This is of some importance, especially in light of recent reports documenting rearranged "suicide" retroviral vectors as a cause of GCV resistance in transduced tumors (55). Virtually all glioma cell lines transduced with vTKiGFP acquired substantial and significant sensitivity to GCV *in vitro* (Fig. 4). Our experimental design based on the use of polyclonal transduced cell populations for cytotoxicity assays supports the hypothesis that vTKiGFP gene transfer, on the average, will express biologically significant levels of TK in a gene-modified cell. Neither the transduction process (with a control retrovector) nor expression of the GFP reporter, on their own, sensitizes cells to GCV (Fig. 4).

Important characteristics of VSVG-pseudotyped retroparticles are their ability to sustain concentration by ultracentrifugation and repeated freeze/thaw without loss of activity. These properties have allowed us to collect retroparticle-conditioned media on a daily basis after tetracycline withdrawal from the 293AP3 producer cell line. Retroparticle-containing medium was frozen and stored until further use. Large volumes of frozen supernatant can be thawed, pooled, and subjected to at least two cycles of centrifugation with efficient retrovector recovery. We concentrated 100 ml of media to a final volume of 0.1 ml (1000× concentration on volume basis). This was accompanied by an 800-fold increase in titer from 2.9 to 2300×10^7 cfu/ml. We noted that supernatant from tetracycline-deprived 293AP3 producer cells could be toxic to target cells if applied repeatedly. We also observed this phenomena with other 293GPG-derived producer cells (data not shown). Interestingly, we observed that concentrated retroparticles, which had been resuspended in serum-free media, did not have this property, although they would be delivered at a MOI higher than that achievable with the unconcentrated supernatant. This suggests that supernatant from tetracycline-deprived 293GPG cells contains toxic constituent(s) that are readily discarded upon concentration procedure.

To test the therapeutic usefulness of this reagent, we used a rodent model of brain cancer. We established C6/lacZ glioma tumors in immunocompetent Sprague Dawley rats and subsequently administered concentrated vTKiGFP retrovector intratumorally. Intratumoral delivery of 9 μl ($\sim 10^8$ retroparticles) of concentrated vTKiGFP retrovector stock did not improve survival of animals who did not subsequently receive GCV. These control rats (tumor+, retrovector+, but no GCV) had a mean survival of 38 days (range, 20–52 days). Postmortem examination of whole-brain tissue sections revealed that efficient and stable tumor-specific gene transfer had occurred (Fig. 7). Transgene expression persisted in the growing tumor as long as rats survived after retrovector administration (data not shown). Examination of surrounding normal brain tissue failed to reveal GFP fluorescence (Fig. 7), suggesting that retrovector integration and expression occurred in tumor cells only and not in mitotically quiescent neurons, as would be expected from a retroviral vector.

Twelve test rats received GCV after tumor-targeted vTKiGFP delivery. Of these, two died shortly (within 2 weeks) following the end of GCV treatment. This "acute" death rate attributable to direct GCV toxicity (~16%) is comparable with that observed by other investigators who administered GCV at equal or lesser doses (7, 42). The mechanism of death is likely related to cytopenia and immunosuppression associated with severe, albeit reversible, bone marrow toxicity. Surviving test rats fully recovered from GCV toxicity ~2 weeks after its completion.

All of the test rats remained alive and well more than 80 days after tumor implantation. Two rats developed symptomatic tumor recurrences and were sacrificed on day 82 after tumor implantation (Fig. 8). Examination of brain tissue sections on these late relapses revealed large tumors with areas of green fluorescence interspaced with GFP-negative tumor cells (Fig. 7). This suggests that recurrence was due in part to growth of untransduced tumor cells, or of tumor cells in which the retrovector was silenced after integration. The presence of GFP+ tumor cells suggests that the GCV regimen was not intensive and/or durable enough to eliminate all transduced tumor cells in these rats. Alternatively, a subset of transduced, TK-expressing cells may have acquired resistance to GCV via some other means. Lastly, the "bystander" effect, especially its immune effector arm, may vary in intensity from animal to animal. This may explain the observed pattern of late relapses, suggesting that that there was an early "suicide/bystander" effect that led to increased survival but that some tumor cells, transduced or not, "escaped" from the bystander effect and eventually led to a recurrence. However, the sum of the suicide and bystander effect was clearly sufficient to enhance survival of a majority of animals (66%) who received vTKiGFP and GCV. Our observed long-term survival rate (>120 days) is substantially greater than that observed after intratumoral injection of TK retroviral producer cells (19) and compares favorably with that obtained with suicide adenovectors (42), including those incorporating tumor-specific promoter elements (22).

In the experimental group, 2 of 12 animals died from GCV toxicity and 2 of 12 succumbed to late tumor recurrences. These data suggest that GCV dose reduction would be desirable to lessen toxicity; however, the duration of treatment may need to be extended to allow elimination of all gene-modified cells. The relatively late recurrences (day 82 after implant), led us to speculate that the "immune" bystander effect may have been mitigated in these two animals. It may be possible to increase the immune response by coadministering immunomodulatory genes (*IL-2* and *granulocyte/macrophage colony-stimulating factor*) with TK such as has been described by others (56). Furthermore, it may be useful to readminister the suicide retrovector to those animals who have residual disease after a cycle of therapy and to repeat this until maximal response has been achieved. However, it is unknown if a specific, and neutralizing, immune response against VSVG-typed retroparticles will be elicited.

This constitutes the first report of *in vivo* delivery of a cell-free retrovector concentrate with tumor-specific, high efficiency gene transfer and expression, with evident biologically significant antitumor activity. We propose that concentrated vTKiGFP retrovector may be of therapeutic value for humans with brain cancer. The high titer of the concentrated reagent would allow intratumor delivery of a useful retrovector dose without the risks of injecting relatively large volumes in a confined space (such as brain). vTKiGFP targeting of a tumor mass *in vivo* should subsequently lead to its regression, and the bystander effect may have a significant impact on the biology of local and distant micrometastatic glioma deposits within the neuropil. This and related therapeutic reagents may also be useful in the treatment of other locally advanced and metastatic malignancies.

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Note Added in Proof

A supplementary test animal that had received vTKiGFP and GCV died on day 125 after tumor implantation. On post mortem, it had a large intracerebral tumor which was GFP negative. All the other test animals (7 of 12) which had received treatment remain alive and well 270 days after tumor implantation.

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PATENT COOPERATION TREATY

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
14226-2PCT		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/CA00/00445	20/04/2000	23/04/1999
International Patent Classification (IPC) or national classification and IPC		
C12N15/00		
Applicant		
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1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 3 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☒ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
20/11/2000	23.07.2001
Name and mailing address of the international preliminary examining authority:	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Wimmer, G Telephone No. +49 89 2399 7347

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/CA00/00445

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-33 as originally filed

Claims, No.:

1-17 with telefax of 10/07/2001

Drawings, sheets:

1/14-14/14 as originally filed

Sequence listing part of the description, pages:

1, filed with the letter of 27.06.2000

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☒ furnished subsequently to this Authority in written form.
- ☒ furnished subsequently to this Authority in computer readable form.
- ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

   ☐ the entire international application.

   ☒ claims Nos. 13 (with respect to Industrial Applicability).

because:

   ☒ the said international application, or the said claims Nos. 13 relate to the following subject matter which does not require an international preliminary examination (*specify*):
   see separate sheet

   ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

   ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

   ☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

   ☐ the written form has not been furnished or does not comply with the standard.

   ☐ the computer readable form has not been furnished or does not comply with the standard.

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

☐ restricted the claims.

☐ paid additional fees.

☐ paid additional fees under protest.

☐ neither restricted nor paid additional fees.

2. ☒ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

☐ complied with.

☒ not complied with for the following reasons:
see separate sheet

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

☒ all parts.

☐ the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	
	No:	Claims	1-17
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-17
Industrial applicability (IA)	Yes:	Claims	1-12, 14-17
	No:	Claims	

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item III

Non-establishment of opinion.

Claim 13 relates to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of this claim (Article 34(4)(a)(i) PCT).

Re Item IV

Lack of unity of invention.

The present patent application refers to methods and entities concerned with retroviral gene delivery into tumor cells.

Specifically, the claims of the application can be grouped as follows:

I) VSV-g pseudotyped retroviral particle for gene delivery into tumor cells, and method employing the same
(Claims 1, 15)

II) Tumor-specific retroviral expression system including a marker gene, and a second gene of interest
(Claims 2-17)

The technical feature (Rule 13.2 PCT) common to these groups is the tumor specific gene expression through retroviral delivery.
This feature, however, does not define a contribution over the prior art, since retroviral gene delivery into tumor cells had been reported extensively in the prior art (see e.g. D1 and references therein). Since a special technical feature as required under Rule 13(2) PCT is therefore lacking, unity of invention is compromized, and the above defined groups constitute two separate inventions.

While applicants allege that "nowhere in Rule 13.2 PCT is there a requirement that a 'special technical feature' be inventive", and therefore claim that the above defined

groups are part of the same invention, the IPEA would like to point out that not only does the the full context of Rule 13 clearly indicate that unity of invention requires a "single general inventive concept" (Rule 13.1), but also that in the present case, the question of whether the technical feature common to the above defined groups be inventive or not does not arise, since it is not novel. Therefore, the common feature does not make any contribution over the prior art (Rule 13.2 PCT) and is insufficient to support unity of invention.

Since, however, the examination of these different inventions poses no excessive effort, no invitation to restrict or to pay additional fees is extended at the moment.

Re Item V

Reasoned statement under Art. 35(2) PCT with regard to novelty, inventive step or industrial applicability.

1) Reference is made to the following documents (the document numbering corresponds to their order of citation in the international search report):

D1: Nalbantoglu J. et al.:'VSV-G pseudotyped retrovector mediates high efficiency in vivo gene transfer in glioma-targeted suicide gene delivery' NEUROLOGY, vol. 52, 12 April, 1999 (1999-04-12), page A425 XP000964616

D2: WO 99 04026 A (CHIRON CORP) 28 January 1999 (1999-01-28)

Novelty under Art. 33(2) PCT.

2) Both documents D1 and D2 disclose subject-matter of claims 1-17.
Document D1 appears to be a disclosure of the invention by the applicants prior to the claimed priority date. D1 discloses the construction of VSV-g pseudotyped retroviral vectors, which include HSV TK as therapeutic gene. Production of viral particles, injection into tumor tissue and treatment with gancyclovir, leading to necrosis of tumors treated with the pseudotyped recombinant virus, are described. Equally, the document cites the use of EGFP as a reporter of provirus transfer and

expression in target cells, and the establishment of a stable producer cell line.

Likewise, document D2 discloses most of the features of the claims. This document describes lentiviral vectors, including flanking LTRs, a packaging signal, a primer building site, and one or more genes of interest (pg. 2). Preferred embodiments include pseudotyping with the VSV-g protein (pgs. 2-3), the use of an Internal Ribosomal Entry Site (pgs. 8-9), and a gene of interest. Examples for the latter are given with marker genes such as GFP (pg. 2), or therapeutic genes. A specific example herein is the expression of HSV TK, which, in combination with treatment with e.g. gancyclovir (pg. 13) leads to specific destruction of cells. The application to tumor tissue (pg. 17) is a preferred embodiment.

Therefore, subject-matter of claims 1-17 is already present in documents D1 and D2, and the claims are not novel.

Industrial Applicability under Art. 33(4) PCT.

3) For the assessment of the present claim 13 on the question whether it is industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Applicants wished to defer dealing with the issues of Novelty and Industrial Applicability to the national phase.

WHAT IS CLAIMED IS:

1. A retroviral particle for delivering a gene to a tumor tissue cell, said retroviral particle being pseudotyped with a vesicular stomatitis virus G (VSV G) protein.

2. A tumor-specific retroviral expression vector comprising a suitable promoter, a retroviral untranslated sequence including a packaging sequence and a primer building site, a cloning site perably linked to an internal ribosomal entry site (IRES), said IRES being operably linked to a first nucleotide sequence encoding a suitable marker, a retroviral 3' long terminal repeat (LTR) sequence, for expressing a second nucleotide sequence inserted in said cloning site.

3. A retroviral expression vector according to claim 2, wherein said second nucleotide sequence comprises a therapeutic gene.

4. A retroviral expression vector according to claim 3, wherein said therapeutic gene comprises a suicide gene.

5. A retroviral expression vector according to claim 3, wherein said suicide gene is TK.

6. A retroviral expression vector according to claim 4, wherein said nucleotide sequence encodes a Herpes simplex virus thimidine kinase.

7. A retroviral expression vector according to claim 5 or 6, wherein said marker comprises a green fluorescent protein (GFP).

8. A retroviral expression vector according to claim 5 or 7, wherein said expression protein is a GFP/TK fusion protein.

9. A plasmid encoding a bicistronic, non-splicing murine retrovector comprising a multiple cloning site (MCS) operably linked to an enhanced green fluorescent (EGFP) reporter (AP2) for transferring a provirus to a target cell and expressing said provirus into said target cell, for co-expressing a nucleotide

sequence inserted into said plasmid with said EGFP reporter within a bicistronic framework.

10. A replication-defective retroviral expression vector comprising a suitable promoter, a retroviral untranslated sequence including a packaging sequence and a primer building site, a multiple cloning site (MCS) operably linked to an internal ribosomal entry site (IRES), said IRES being operably linked to a first nucleotide sequence encoding a suitable marker, a retroviral 3' long terminal repeat (LTR) sequence, for expressing a second DNA sequence inserted in said MCS.

11. An expression vector according to claim 10, wherein said marker comprises an enhanced green fluorescent protein (EGFP).

12. An expression vector according to claim 10, wherein said promoter comprises a CMV promoter.

13. A method for treating a tumor, the method comprising administering to a mammal suspected of having a tumor a retroviral expression vector comprising a first nucleotide sequence, said first nucleotide sequence being therapeutic, and a second nucleotide sequence encoding a marker, said first and second nucleotide sequences being co-dominantly expressed, and administering to said mammal a nucleobase analog.

14. A method for detecting *in vivo* a genetically modified cell with an expression vector according to claim 9 to a tumor tissue cell of a mammal, the method comprising administering a retroviral expression vector comprising a first nucleotide sequence encoding a retrovirus and a second nucleotide sequence encoding a marker, said first and second nucleotide sequences being co-dominantly expressed, and detecting the expression of said second nucleotide sequence by using one of fluorescence microscopy and flow cytometry techniques.

15. A method for producing a retroviral particle according to claim 1, the method comprising stably transfecting a suitable cell line with the expression vector of claim 9.

16. A method for producing retroviral particles, the method comprising transfecting a suitable cell line with the expression vector of claim 10 and transfecting said cell line with a drug resistance plasmid.

17. The cell line obtained by the method according to claim 14.

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark Office, PCT
2011 South Clark Place Room CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 08 January 2001 (08.01.01)	
International application No. PCT/CA00/00445	**Applicant's or agent's file reference** 14226-2PCT
International filing date (day/month/year) 20 April 2000 (20.04.00)	**Priority date** (day/month/year) 23 April 1999 (23.04.99)
Applicant GALIPEAU, Jacques	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

20 November 2000 (20.11.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Nestor Santesso
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

CA0000445

Form PCT/IB/331 (July 1992)

PATENT COOPERATION TREATY

by fax and post

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

PCT

To:

SWABEY OGILVY RENAULT
1981, Avenue McGill College
Bureau 1600
Montréal, Québec H3A 2Y3
CANADA

FAX: (514) 288-8389

RECEIVED
JUL 27 2001

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	23.07.2001
Applicant's or agent's file reference 14226-2PCT FC	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/CA00/00445	20/04/2000	23/04/1999

Applicant
CENTRE FOR TRANSLATIONAL RESEARCH IN CANCER et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Büchler, S Tel.+49 89 2399-8090

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
14226-2PCT		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/CA00/00445	20/04/2000	23/04/1999
International Patent Classification (IPC) or national classification and IPC C12N15/00		
Applicant CENTRE FOR TRANSLATIONAL RESEARCH IN CANCER et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 3 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☒ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
20/11/2000	23.07.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Wimmer, G Telephone No. +49 89 2399 7347

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/CA00/00445

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-33 as originally filed

Claims, No.:

1-17 with telefax of 10/07/2001

Drawings, sheets:

1/14-14/14 as originally filed

Sequence listing part of the description, pages:

1, filed with the letter of 27.06.2000

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☒ furnished subsequently to this Authority in written form.
- ☒ furnished subsequently to this Authority in computer readable form.
- ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

   ☐ the entire international application.

   ☒ claims Nos. 13 (with respect to Industrial Applicability).

because:

   ☒ the said international application, or the said claims Nos. 13 relate to the following subject matter which does not require an international preliminary examination (*specify*):
   see separate sheet

   ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

   ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

   ☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

   ☐ the written form has not been furnished or does not comply with the standard.

   ☐ the computer readable form has not been furnished or does not comply with the standard.

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

☐ restricted the claims.

☐ paid additional fees.

☐ paid additional fees under protest.

☐ neither restricted nor paid additional fees.

2. ☒ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

☐ complied with.

☒ not complied with for the following reasons:
see separate sheet

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

☒ all parts.

☐ the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	
	No:	Claims	1-17
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-17
Industrial applicability (IA)	Yes:	Claims	1-12, 14-17
	No:	Claims	

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item III

Non-establishment of opinion.

Claim 13 relates to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of this claim (Article 34(4)(a)(i) PCT).

Re Item IV

Lack of unity of invention.

The present patent application refers to methods and entities concerned with retroviral gene delivery into tumor cells.

Specifically, the claims of the application can be grouped as follows:

I) VSV-g pseudotyped retroviral particle for gene delivery into tumor cells, and method employing the same
(Claims 1, 15)

II) Tumor-specific retroviral expression system including a marker gene, and a second gene of interest
(Claims 2-17)

The technical feature (Rule 13.2 PCT) common to these groups is the tumor specific gene expression through retroviral delivery.
This feature, however, does not define a contribution over the prior art, since retroviral gene delivery into tumor cells had been reported extensively in the prior art (see e.g. D1 and references therein). Since a special technical feature as required under Rule 13(2) PCT is therefore lacking, unity of invention is compromized, and the above defined groups constitute two separate inventions.

While applicants allege that "nowhere in Rule 13.2 PCT is there a requirement that a 'special technical feature' be inventive", and therefore claim that the above defined

groups are part of the same invention, the IPEA would like to point out that not only does the the full context of Rule 13 clearly indicate that unity of invention requires a "single general inventive concept" (Rule 13.1), but also that in the present case, the question of whether the technical feature common to the above defined groups be inventive or not does not arise, since it is not novel. Therefore, the common feature does not make any contribution over the prior art (Rule 13.2 PCT) and is insufficient to support unity of invention.

Since, however, the examination of these different inventions poses no excessive effort, no invitation to restrict or to pay additional fees is extended at the moment.

Re Item V

Reasoned statement under Art. 35(2) PCT with regard to novelty, inventive step or industrial applicability.

1) Reference is made to the following documents (the document numbering corresponds to their order of citation in the international search report):

D1: Nalbantoglu J. et al.:'VSV-G pseudotyped retrovector mediates high efficiency in vivo gene transfer in glioma-targeted suicide gene delivery' NEUROLOGY, vol. 52,12 April,1999 (1999-04-12), page A425 XP000964616

D2: WO 99 04026 A (CHIRON CORP) 28 January 1999 (1999-01-28)

Novelty under Art. 33(2) PCT.

2) Both documents D1 and D2 disclose subject-matter of claims 1-17. Document D1 appears to be a disclosure of the invention by the applicants prior to the claimed priority date. D1 discloses the construction of VSV-g pseudotyped retroviral vectors, which include HSV TK as therapeutic gene. Production of viral particles, injection into tumor tissue and treatment with gancyclovir, leading to necrosis of tumors treated with the pseudotyped recombinant virus, are described. Equally, the document cites the use of EGFP as a reporter of provirus transfer and

expression in target cells, and the establishment of a stable producer cell line.

Likewise, document D2 discloses most of the features of the claims. This document describes lentiviral vectors, including flanking LTRs, a packaging signal, a primer building site, and one or more genes of interest (pg. 2). Preferred embodiments include pseudotyping with the VSV-g protein (pgs. 2-3), the use of an Internal Ribosomal Entry Site (pgs. 8-9), and a gene of interest. Examples for the latter are given with marker genes such as GFP (pg. 2), or therapeutic genes. A specific example herein is the expression of HSV TK, which, in combination with treatment with e.g. gancyclovir (pg. 13) leads to specific destruction of cells. The application to tumor tissue (pg. 17) is a preferred embodiment.

Therefore, subject-matter of claims 1-17 is already present in documents D1 and D2, and the claims are not novel.

Industrial Applicability under Art. 33(4) PCT.

3) For the assessment of the present claim 13 on the question whether it is industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Applicants wished to defer dealing with the issues of Novelty and Industrial Applicability to the national phase.

WHAT IS CLAIMED IS:

1. A retroviral particle for delivering a gene to a tumor tissue cell, said retroviral particle being pseudotyped with a vesicular stomatitis virus G (VSV G) protein.

2. A tumor-specific retroviral expression vector comprising a suitable promoter, a retroviral untranslated sequence including a packaging sequence and a primer building site, a cloning site preferably linked to an internal ribosomal entry site (IRES), said IRES being operably linked to a first nucleotide sequence encoding a suitable marker, and a retroviral 3' long terminal repeat (LTR) sequence, for expressing a second nucleotide sequence inserted in said cloning site.

3. A retroviral expression vector according to claim 2, wherein said second nucleotide sequence comprises a therapeutic gene.

4. A retroviral expression vector according to claim 3, wherein said therapeutic gene comprises a suicide gene.

5. A retroviral expression vector according to claim 4, wherein said suicide gene is TK.

6. A retroviral expression vector according to claim 4, wherein said second nucleotide sequence encodes a Herpes simplex virus thimidine kinase.

7. A retroviral expression vector according to claim 5 or 6, wherein said marker comprises a green fluorescent protein (GFP).

8. A retroviral expression vector according to claim 5 or 7, wherein said a first and second nucleotide sequences are combined to encode a GFP/TK fusion protein.

9. A plasmid encoding a bicistronic, non-splicing murine retrovector comprising a multiple cloning site (MCS) operably linked to an enhanced green

fluorescent (EGFP) reporter (AP2) for transferring a provirus to a target cell and expressing said provirus into said target cell, for co-expressing a nucleotide sequence inserted into said plasmid with said EGFP reporter within a bicistronic framework.

10. A replication-defective retroviral expression vector comprising a suitable promoter, a retroviral untranslated sequence including a packaging sequence and a primer building site, a multiple cloning site (MCS) operably linked to an internal ribosomal entry site (IRES), said IRES being operably linked to a first nucleotide sequence encoding a suitable marker and, a retroviral 3' long terminal repeat (LTR) sequence, for expressing a second DNA sequence inserted in said MCS.

11. An expression vector according to claim 10, wherein said marker comprises an enhanced green fluorescent protein (EGFP).

12. An expression vector according to claim 10, wherein said promoter comprises a CMV promoter.

13. A method for treating a tumor, the method comprising administering to a mammal suspected of having a tumor a retroviral expression vector comprising a first nucleotide sequence, said first nucleotide sequence being therapeutic, and a second nucleotide sequence encoding a marker, said first and second nucleotide sequences being co-dominantly expressed, and administering to said mammal a nucleobase analog.

14. A method for detecting *in vivo* a genetically modified cell with an expression vector according to claim 9 to a tumor tissue cell of a mammal, the method comprising administering a retroviral expression vector comprising a first nucleotide sequence encoding a retrovirus and a second nucleotide sequence encoding a marker, said first and second nucleotide sequences being co-dominantly expressed, and detecting the expression of said second nucleotide sequence by using one of fluorescence microscopy and flow cytometry techniques.

15. A method for producing a retroviral particle according to claim 1, the method comprising stably transfecting a suitable cell line with the expression vector of claim 9.

16. A method for producing retroviral particles, the method comprising transfecting a suitable cell line with the expression vector of claim 10 and transfecting said cell line with a drug resistance plasmid.

17. The cell line obtained by the method according to claim 14.

AMENDED SHEET